asked for clarification about the word "molecules"; and claims 1 and 9 are objected to for the temperature for the cycle primer extension. We have addressed all of these points by amending claims 1, 2, 8, and 9 above, and believe that this should be sufficient to overcome this rejection. However, if the Examiner has alternative claim language to suggest, the Examiner is invited to contact applicant's representative to discuss it.

Claims 1-8 were rejected under 35 U.S.C. §102(b) as anticipated by Fuller (U.S. Patent 5,432,065). Claims 9-11 were rejected under 35 U.S.C. §103(a) as obvious over Fuller in view of Ruano (U.S. Patent 5,427,911). We traverse these rejections for the following reasons.

Regarding the primary reference Fuller, we note that, while Fuller mentions a glycerol level of 10 – 50% v/v and an ethylene glycol level of 20% v/v, there is no data whatsoever to support this, and, in fact, there is evidence in the prior art that applying the teachings of Fuller does not generate amplification products that are sequence-specific. Fuller did not achieve amplification of useful PCR products which are sequence-specific with the 40% glycerol or ethylene glycol as additives. By contrast, the inventors of the subject application discovered that 40% glycerol is actually detrimental for low temperature PCR with high polymerization specificity using moderately thermostable DNA polymerases; the inventors also unexpectedly found that the use of 10-20% glycerol with three DNA polymerases is effective for low temperature polymerase chain reactions.

Fuller's patent is not technically accurate, at least, not in its entirety, and this has been recognized by others in this area of art. For instance, Fuller's examples and claims focus on a method for performing a cycled primer extension reaction using about 40% (v/v) glycerol (final concentration) to lower the melting temperature of the template DNA to carry out the extension at temperatures below 80° C, or less than 95°C using Bca or Klenow DNA polymerase. Notably, Fuller did not present any experimental data or results to demonstrate that the cycle amplification of the template as proposed in the patent was indeed successful in generating DNA fragments of the size identical to the target template and that the amplification products obtained were sequence-specific.

In 1999, Iakobashvili and Lapidot (Nucleic Acids Research 1999;26:1566-1568, a copy of which was submitted to the Examiner in the Information Disclosure Statement

mailed April 18, 2002) in their attempt to find an applicable method for low temperature cycled PCR, made the following comments on the Fuller patent:

> Klenow fragment of DNA polymerase I from *Escherichia coli* (Klenow polymerase), as well as other thermolabile DNA polymerases, were used for PCR in the early days of this method by fresh addition to each cycle. Introduction of thermostable enzymes added much convenience to PCR, and Klenow polymerase was not applied further in PCR. Fuller (*US Patent 5,432,065*), using glycerol and ethylene glycol as buffer components, developed the ideology of low temperature PCR (LT-PCR); the additives should decrease DNA melting temperature (T*m*) and stabilize thermolabile polymerases without inhibiting their enzymatic activity. Cycling LT-PCR with a moderately-thermostable Bca DNA polymerase was demonstrated in the presence of 40% glycerol, whereas the stability of Klenow polymerase in the presence of >40% glycerol (half-life of 3 min at 65°C in 40% glycerol) was not sufficient for cycling PCR.

In the Fuller patent, the optimal conditions given for low temperature PCR are 40% glycerol, a temperature below 80° C and a DNA polymerase selected form the group consisting of Bca and Klenow. Technically speaking, however, the enzymatic activity of Klenow is totally abolished after 3 min at temperature between 70° C and 80° C. The "readable signal intensities" after LT-PCR with Klenow are in fact non-specific smears, not true PCR products that can be confirmed with DNA sequencing. This was correctly pointed out by Iakobashvili and Lapidot (Nucleic Acids Research 1999;26:1566-1568). Iakobashvili and Lapidot did demonstrate cycling LT-PCR with Bca DNA polymerase (one of the polymerases included in our claims) in the presence of 40% glycerol, ***but could not put it into practice to generate useful template-specific PCR products***. Thus, someone having ordinary skill in this art—such as Iakobashvili and Lapidot—trying to put Fuller's teachings into practice, would be unable to generate cycle amplification products that are sequence-specific and target specific. In summary, the methods taught by Fuller did not teach a method to produce low temperature amplification products that are sequence-specific or target-specific. And it is well recognized in the art that the cycle amplification products are not useful and of no value unless they are sequence-specific and target specific.

In addition, there is not data supporting Fuller's statement that ..."applicant has determined that the melting point of DNA decreased by 0.5 degrees C for each 1% concentration of glycerol or ethylene...." . Scientifically, it is **not possible** to produce any

perceptible effects on the melting temperature of DNA by the addition of 1% of glycerol in an enzymatic mixture because the magnitude of lowering of the melting point and the concentration of the additives are not in a straight linear proportional relationship from zero to 100% of additives. The statement in column 2, lines 3-21 of Fuller that "the invention features a method for performing a cycled primer extension reaction by contacting a template DNA with a primer in the presence of between 10 and 50% (v/v) glycerol or ethylene glycol...", would not have been anticipatory of our own invention, for if someone were to follow Fuller's recommendations in practice to calculate a melting point for low temperature PCR, a 10% glycerol in the PCR reaction mixture would reduce the usually required melting temperature of 95° C by 5° C (i.e. to 90° C). This is clearly too high a temperature for any non-heat-resistant DNA polymerases, such as Bca or Klenow as recommended in the patent, to survive in the PCR process. Therefore, Fuller's misleading teaching on the concentrations of glycerol or ethylene glycol other than 40% could not enable the non-thermostable DNA polymerases selected to perform PCR.

Our invention differs from the Fuller patent in that our invention concerns low temperature cycle extension of DNA with high polymerization specificity. We have amended independent claims 1 and 9 to specify that the DNA polymerase has proofreading 3'-5' exonuclease activity during DNA primer extension over a template, such that the DNA polymerase functions to excise mismatched nucleotides from the 3' terminus of the DNA strand at a faster rate than the rate at which the DNA polymerase functions to remove nucleotides matched correctly with nucleotides of the template. Independent claims 22 and 30 recite that the DNA polymerase during dye-labeled terminator automated DNA cycle sequencing reduces the innate selective discrimination against the incorporation of fluorescent dye-labeled ddCTP and fluorescent dye-labeled ddATP. None of the DNA polymerases described by Fuller possess these characteristics, which are completely unique to our invention. On this basis alone our claims are distinguishable over Fuller, alone or in combination with Ruano.

Unlike Fuller, we have a method whereby specific DNA fragments identical to the double-stranded DNA target template or complementary to a single-stranded DNA target can be amplified at temperatures cycling below 80° C, even using primers of less

than 30 bp long. It has been shown in the art that, although glycerol or ethylene glycol can lower the melting temperature of DNA, it also inactivates the specific 5'-3' polymerization activity of the moderately thermostable DNA polymerases at concentrations higher than 20%. Therefore, we have developed a system for low temperature cycle extension of DNA with high polymerization specificity, using a moderately thermostable DNA polymerase—avoiding the misleading Fuller teaching of incorporating a high concentration of 40% glycerol in the reaction mixture. The novelty of our invention is to use a unique moderately thermostable DNA polymerase of a *Bacillus* species to perform low temperature cycle amplification of DNA with high polymerization specificity in a low (10-20%) concentration of glycerol or ethylene glycol.

In summary, then, someone having ordinary skill in this art, and having Fuller in hand, would not have reasonably found our invention described therein. Fuller is not an anticipating disclosure of claims 1-8, nor would it be usefully combined with the Ruano patent to render our claims 9-11 obvious.

Regarding the Ruano patent (US Patent 5,427,911), its disclosure relates to coupled amplification and sequencing of DNA, using a thermally stable enzyme, e.g., Taq polymerase enzyme, preferably highly purified. The amplification involves a series of cycling steps ("thermal cycling") including denaturation at 90° to 95° C, preferably 94° C, annealing at 45° C to 65° C (the temperature depends on the target), preferably 57° C, and extension at 65° C to 75° C, preferably 72° C. (Column 6, lines 15-20). It is not a low temperature amplification or sequencing of DNA. As emphasized in its Abstract, the invention of Ruano concerns a process for sequencing DNA segments by introducing **specific oligonucleotide primers** annealing to the 3' end of both complementary strands. This patent in fact strengthens the unobviousness of our invention in that a person having ordinary skill in the art to which said subject matter pertains, e.g. an inventor at Yale University Gualberto Ruano, did not recognize and use a low temperature amplification and sequencing for specific oligonucleotide primer extensions.

The unobviouness of the instant invention is further strengthened by the publication of Iakobashvili and Lapidot (mentioned above) who initially relied on the Fuller patent to design a low temperature PCR system, but failed. Instead, they used

another denaturing agent, proline, to achieve a usable low temperature PCR for primers over 30 bp long, but with low specificity when 20-25 bp primers are used. Our invention can be used for extending oligonucleotide primers of various lengths, even for those of 20-25 bp, or shorter, with high polymerization specificity.

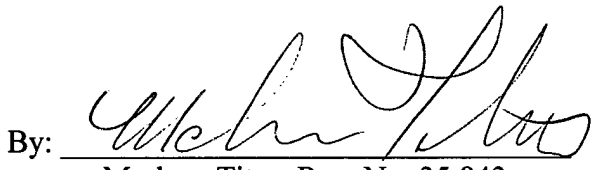
Lastly, like Fuller, Ruano does not teach the unique DNA polymerases that are required by all of the claims now pending—that the DNA polymerase has proofreading 3'-5' exonuclease activity during DNA primer extension over a template, such that the DNA polymerase functions to excise mismatched nucleotides from the 3' terminus of the DNA strand at a faster rate than the rate at which the DNA polymerase functions to remove nucleotides matched correctly with nucleotides of the template, and/or that the DNA polymerase during dye-labeled terminator automated DNA cycle sequencing reduces the innate selective discrimination against the incorporation of fluorescent dye-labeled ddCTP and fluorescent dye-labeled ddATP. Ruano does not teach or suggest such DNA polymerases, and on this basis alone our claims are distinguishable over Fuller combined with Ruano.

Thus, Ruano does not make up for the deficiencies of Fuller, and in fact is not combinable with Fuller to achieve our invention.

For all of these reasons, we request that the two art rejections be withdrawn.

In summary, all of the Examiner's outstanding rejections and objections have been addressed, and the application is believed to be in allowable form. Notice to that effect is earnestly solicited. No amendment made was related to the statutory requirements of patentability unless expressly stated herein, and no amendment made was for the purpose of narrowing the scope of any claim unless we argued above that such amendment was made to distinguish over a particular reference or combination of references.

If the Examiner has any questions or would like to make suggestions as to claim language, she is encouraged to contact Marlana K. Titus at (301) 924-9600.

By: 
Marlana Titus, Reg. No. 35,843

Nash & Titus, LLC
3415 Brookeville Road, Suite 1000
Brookeville, Maryland 20833
(301) 924-9600

MARKED-UP VERSION OF CLAIMS AS AMENDED ABOVE

1. (Amended) A method for extending [a] an oligonucleotide primer or a pair of oligonucleotide primers using an enzymatic cycle primer extension reaction at temperatures between about 45°C and about 65°C, and a melting temperature of about 70°C [below about 80°C], comprising the step of mixing a template DNA with a primer or a pair of primers and a natural or a modified form of a moderately thermostable DNA polymerase from an organism selected from the group consisting of *Bacillus stearothermophilus*, *Bacillus caldotenax* and *Bacillus caldolyticus*, wherein the DNA polymerase has proofreading 3'-5' exonuclease activity during DNA primer extension over a template, such that the DNA polymerase functions to excise mismatched nucleotides from the 3' terminus of the DNA strand at a faster rate than the rate at which the DNA polymerase functions to remove nucleotides matched correctly with nucleotides of the template, in a solution containing between about 10% and about 20% (v/v) glycerol, ethylene glycol, or a mixture [thereof] of glycerol and ethylene glycol, under conditions that the cycle reaction temperature fluctuates between a melting temperature of about 70°C and an annealing temperature of about 37°C, so that the DNA polymerase repeatedly extends the primer or pair of primers.

2. (Amended) The method of claim 1, wherein the glycerol, ethylene glycol, or a mixture [thereof] of glycerol and ethylene glycol is present in about 15% (v/v).

8. (Amended) The method of claim 1, wherein [molecules of a single primer] oligonucleotide primers of various lengths are extended with specific nucleotide terminations in the presence of ddNTPs or their analogs for cycle sequencing.

9. (Amended) A method for extending [the molecules of a] an oligonucleotide primer annealed to a DNA template for direct cycle sequencing of in vitro amplified double-stranded DNA products without prior isolation or purification, using an enzymatic

cycle primer extension reaction at temperatures between about 45°C and about 65°C, and a melting temperature below about 80°C, comprising the steps of:

(i) mixing diluted crude amplified reaction product with an excess amount of a sequencing primer, the four standard ddNTP terminators or their corresponding analogs, a native or modified form of a moderately thermostable DNA polymerase selected from the group consisting of *Bacillus stearothermophilus*, *Bacillus caldotenax* and *Bacillus caldolyticus*, wherein the DNA polymerase has proofreading 3'-5' exonuclease activity during DNA primer extension over a template, such that the DNA polymerase functions to excise mismatched nucleotides from the 3' terminus of the DNA strand at a faster rate than the rate at which the DNA polymerase functions to remove nucleotides matched correctly with nucleotides of the template, a suitable concentration of dNTPs, and a composition comprising a buffer in a solution containing about 10% to about 20% of glycerol, ethylene glycol, or a mixture [thereof] of glycerol and ethylene glycol, and

(ii) effecting cycle primer extension reaction(s) at a temperature below about 80°C for a sufficient number of times to extend the sequencing primer molecules to desired lengths terminated specifically by ddNTPs or their corresponding analogs.